Part #26

Patent Docket P1363R1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Deborah Ann Ansaldi Serial No.: 09/320,100 Filed: 26 MAY 1999 For: SEPARATION OF POLYPEPTIDE MONOMERS	Group Art Unit: 1642 Examiner: Anne Holleran CERTIFICATE OF FACSIMILE TRANSMISSION February , 2003 : Date of Transmission I hereby certify that this correspondence, consisting of Amendment, Declaration, and Amendment Transmittal, is being facsimile transmitted to the Assistant Commissioner of Patents, Washington, D.C. 20231. Janet E. Hasak

Considered
RLH
5/5/03

DECLARATION UNDER 37 CFR §1.132

Assistant Commissioner of Patents
Washington, D.C. 20231

I, Philip Lester, do hereby declare and say as follows:

1. I am a co-inventor on the above patent application.

2. I have reviewed the Office Action dated 11/18/02 and the references cited with it with respect to the present application.

3. I believe the applicants have supplied enough information in the present application to allow a scientist versed in protein purification technology to perform purification of polypeptide monomers away from their corresponding dimers and multimers as claimed. The pH ranges specified in the claims are not too broad. In practice, the skilled practitioner would select a pH based on the isoelectric point (pI) of the target protein. For instance, if the target protein has a pI of 7, one would select a pH of less than 7 for cation-exchange chromatography, and a pH of greater than 7 for anion-exchange chromatography. Note that this is a starting point for all ion-exchange separations, and one routinely determines the pH at which the protein binds. This determination does not entail an excessive amount of experimentation in order to develop an effective chromatographic

separation. One skilled in the art can easily determine the stability of a protein at various pH levels as part of the consideration for purification, for example, by testing denaturation or activity loss, and the present claims cover these acceptable pH ranges in specifying a pH of 4-7 or 6-9. A basic reference in support of this approach is the Pharmacia handbook, Ion Exchange Chromatography, Principles and Methods, 3rd edition, pp. 51-53 (April 1991), attached as Exhibit A.

4. Moreover, the examples in the present application demonstrate the purification method using representative proteins having a broad range of physico-chemical characteristics. In particular, BSA and IgG, the proteins exemplified in the present application, were carefully chosen as representative examples of proteins to use since they vary in molecular weight, pI, and structure. The molecular weight of BSA is about 68 kD, it has a relatively low pI (about 4.9), and it is a single-chain protein, while the molecular weight of IgG is about 150 kD, and it has a higher pI (about 8) and is composed of 4 subunits linked by disulfide bonds. These two proteins show that the claimed purification method is operable with a broad range of proteins having different physio-chemical properties.

5. The Arndt reference cited by the Examiner (Biochemistry, 37: 12918-12926 (1998)) does not apply to the present claims. It describes a phenomenon of a protein that forms monomers or dimers depending on the pH and the ionic strength of the solution, as well as the presence or absence of antigen. The authors employ analytical size-exclusion chromatography (SEC) to determine the amount of monomer and dimer in their experiments, and they use SEC to separate monomer from dimer. SEC is a well established method for this type of separation. The authors do not use ion-exchange chromatography to achieve this separation as presently claimed, but rather supports the fact, disclosed in the background section of the present application on page 2, lines 2-3, that SEC was commonly used as of the filing date to separate monomers from multimers.

6. The Yang *et al.* article cited by the Examiner (Molecular Immunology, 32: 873-881; pages 876-877, bridging paragraph (1995)) supposedly shows how one skilled in the art was 'unsuccessful' at separating monomers of FV/TNF from dimers and multimers of FV/TNF using ion-exchange chromatography. There is simply no indication in this paper that the authors

were seeking to separate monomers from dimers and multimers using ion-exchange chromatography. Based on the information provided in Yang *et al.*, I can reach two conclusions. Either (1) they did achieve separation of monomers, dimers, and trimers from each other during the ion-exchange chromatography but failed to recognize or appreciate this, and/or their pooling criteria were so broad that these forms were re-mixed into a single fraction, or (2) the monomer, dimers and trimers were aggregated together and therefore could not be separated during ion-exchange chromatography, and eluted as a single peak. In my opinion, the latter is the most likely to have occurred.

7. Yang *et al.* refer to a paper (Chaudary, V. *et al.*, (1989), Nature 339, 394-397), attached hereto as Exhibit B, that describes a similar method from which Yang *et al.* derived their method. The Chaudary article describes the elution gradient in more detail, including slope, flow rate, fraction volume, etc. Chaudary claims to have separated monomers from "high-molecular weight aggregates," which eluted at higher ionic strength using ion-exchange chromatography (p. 395, col. 1). This is consistent with what others have reported in the literature, which is not what is being claimed in this application. Chaudary makes no mention of dimers, trimers, and multimers. In fact, the authors use a TSK 250 gel-filtration column to further purify the monomer--from what, they do not say--yet they say that "the chimaeric protein eluted as a symmetrical peak at the location expected for a 65 K protein (data not shown)" (page 395, col. 1-2). I can conclude two things from the results reported in Chaudary: either (1) there were no dimers, trimers, or multimers in the load, or (2) if these forms were present, Chaudary achieved only partial success in their separation and needed a second purification step, in this case SEC, to remove these forms.

8. The teaching of Yang *et al.* leaves several critical questions unanswered:

a) What were they separating? It appears that they were separating the FV/TNF from host cell proteins. Indeed, the results presented in Figure 2 show that the described ion-exchange chromatography yielded a mixture of monomers, dimers, and trimers. The authors go on to state that the trimer form is the "active functional form" (*see* p. 878, col. 2, top, last sentence). They also imply that the other forms are active, suggesting that they were not attempting to purify desired

monomers from such trimers, as claimed in the present application. Thus, the authors report successful purification of a mixture of monomers, dimers, and trimers from unrelated bacterial proteins. In view of the state of the literature at that time (1995), they would likely have used SEC to purify the monomer from its dimers and trimers if they desired to obtain such separation.

b) As mentioned above in paragraph 6 as the second possibility, the feedstock may have contained only aggregate that would elute as a single peak: i.e., the feedstock could have contained no monomer, dimer, or multimer, but only higher-order aggregates. The only analytical method used to determine the x-mer forms was SDS-PAGE. Non-reduced SDS-PAGE can disrupt all non-disulfide-linked aggregates, and reduced SDS-PAGE can reduce all disulfide-linked aggregates. This could lead to results similar to those shown in Figure 2, gels A and B. Analytical SEC could answer this question, but it was not used in Yang *et al.*

9. In conclusion, the Arndt reference has no relevance to separation or purification conditions, and the Yang reference is completely ambiguous as to what was separated and certainly the authors had no appreciation for separation of monomers, dimers, and multimers from each other. Further, the examples of this application show purification using representative proteins with a broad array of properties. Hence, I believe that one skilled in the art would have sufficient information to have been able, as of the filing date of the present application, to perform successfully the purification method using the polypeptides and conditions as currently claimed.

10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

14 February 2003
Date

Philip Lester

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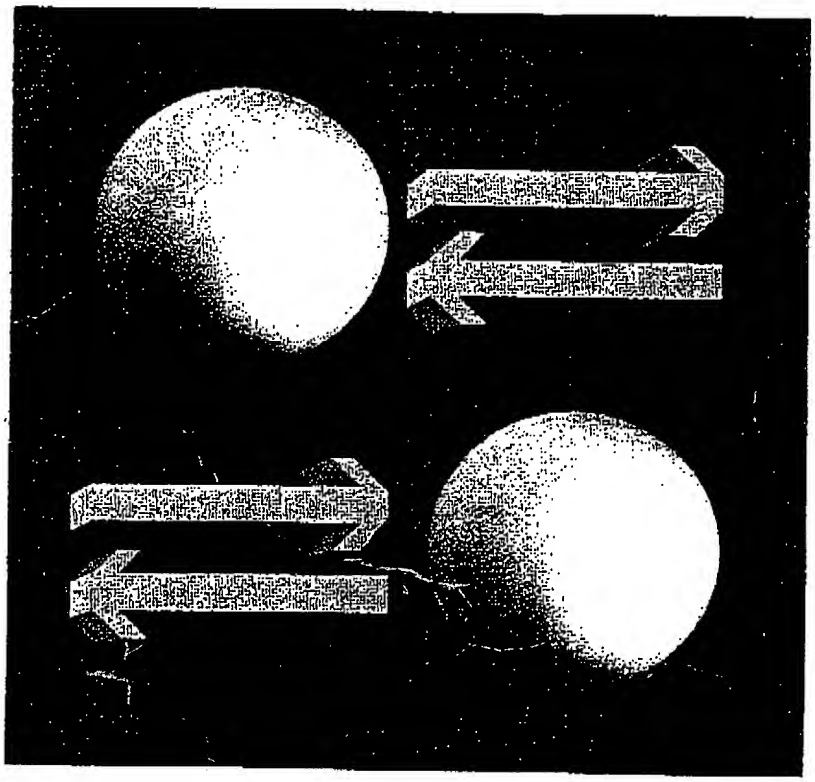
Ion Exchange Chromatography



Principles and Methods

3 rd edition
[18-1022-19]

Pharmacia
Pharmacia LKB Biotechnology

EXHIBIT A

Ion Exchange

ISBN 91 970490-3-4

Choice of exchanger group

Substances are bound to ion exchangers when they carry a net charge opposite to that of the ion exchanger. This binding is electrostatic and reversible.

In the case of substances which carry only one type of charged group the choice of ion exchanger is clear-cut. Substances which carry both positively and negatively charged groups, however, are termed amphoteric and the net charge which they carry depends on pH (Fig. 24). Consequently at a certain pH value an amphoteric substance will have zero net charge. This value is termed the isoelectric point (pI) and at this point substances will bind to neither anion or cation exchangers.

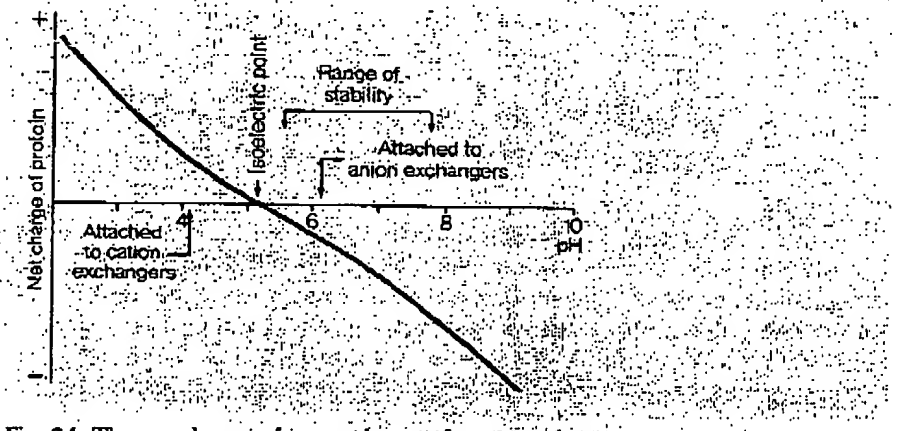


Fig. 24. The net charge of a protein as a function of pH.

The pH ranges in which the protein is bound to anion or cation exchangers and an arbitrary range of stability are shown.

The pH of the buffer thus determines the charge on amphoteric molecules during the experiment. In principle therefore, one could use either an anion or a cation exchanger to bind amphoteric samples by selecting the appropriate pH. In practice however, the choice is based on which exchanger type and pH give the best separation of the molecules of interest, within the constraints of their pH stability.

Methods for determining the optimum pH and corresponding ion exchanger type are discussed later in this chapter.

Many biological macromolecules become denatured or lose activity outside a certain pH range and thus the choice of ion exchanger may be limited by the stability of the sample. This is illustrated in Figure 24. Below its isoelectric point a protein has a net positive charge and therefore can adsorb to cation exchangers. Above its pI the protein has a net negative charge and can be adsorbed to anion exchangers. However, it is only stable in the range pH 5–8 and so an anion exchanger has to be used.

In summary:

1. If the sample components are most stable below their pI's, a cation exchanger should be used.
2. If they are most stable above their pI's, an anion exchanger should be used.
3. If stability is high over a wide pH range on both sides of pI, either type of ion exchanger can be used.

Determination of starting conditions

The isoelectric point

The starting buffer pH is chosen so that substances to be bound to the exchanger are charged. The starting pH should be at least 1 pH unit above the isoelectric point for anion exchangers or at least 1 pH unit below the isoelectric point for cation exchangers to facilitate adequate binding. Substances begin to dissociate from ion exchangers about 0.5 pH units from their isoelectric points at ionic strength 0.1 M (16).

There are comprehensive lists of isoelectric points determined for proteins (17, 18) which can be useful in the design of ion exchange experiments.

If the isoelectric point of the sample is unknown, a simple test can be performed to determine which starting pH can be used.

Test-tube method for selecting starting pH

1. Set up a series of 10 test-tubes (15 ml).
2. Add 0.1 g Sephadex ion exchanger or 1.5 ml Sepharose or Sephacel ion exchanger to each tube.
3. Equilibrate the gel in each tube to a different pH by washing 10 times with 10 ml of 0.5 M buffer (see page 61 for choice of buffers for ion

exchange). Use a range of pH 5–9 for anion and pH 4–8 for cation exchangers, with 0.5 pH unit intervals between tubes.

4. Equilibrate the gel in each tube at a lower ionic strength (0.05 M for Sephadex or 0.01 M for Sepharose and Sephacel ion exchangers) by washing 5 times with 10 ml of buffer of the same pH but lower ionic strength.
5. Add a known constant amount of sample to each tube.
6. Mix the contents of the tubes for 5–10 minutes.
7. Allow the gel to settle.
8. Assay the supernatant for the substance of interest. The results may appear as shown in Figure 25(a).

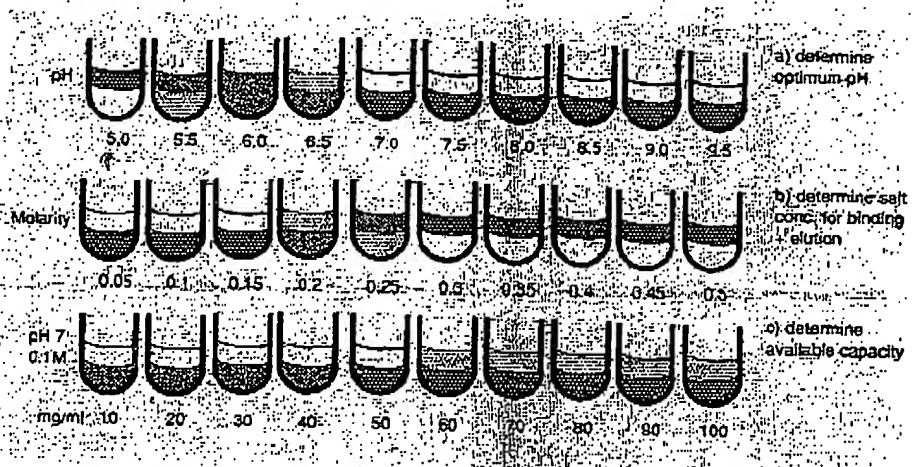


Fig. 25. Test-tube methods for selecting ion exchange conditions.

The pH to be used in the experiment should allow the substance to be bound, but should be as close to the point of release as possible. If too low (or high) a pH is chosen, elution may become more difficult and high salt concentrations may have to be used. In Figure 25 the buffer chosen should be pH 7.0.

Electrophoretic titration curves (ETC)

While information on the pI of the sample components gives valuable indications concerning the choice of starting conditions, it does not give a picture of how the charge on the molecules varies with pH (Fig. 24), nor

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EXHIBIT B

This might explain the problems so far encountered in the studies of binding labelled peptides to MHC on intact cells[2-8]. At the concentration of peptide 17–29 (100 ng ml^{-1}) that we have routinely used, the ratio of binding to DR1 versus the other binding is favourable. The data summarized in Fig. 3*a* also indicate that the binding of peptide 17–29 to EHM cells approaches saturation at a level of class II-dependent binding of about 7×10^4 molecules per cell; at this level ~1% of the peptide offered is bound. This binding involves at most 15% of all DR molecules that are found on the membrane of an EBV-transformed B-cell line[17], which is in agreement with the fact that the majority of class II MHC binding sites are occupied by endogenous peptides[18].

Figure 3*b* shows the kinetics of binding of peptide 17–29 to EHM cells; maximum binding is achieved in 45 min, which is much shorter than the 6 hours or longer needed for maximum binding of peptides to purified class II MHC molecules[2,3]. In fact, in the same system of peptide 17–29 and DR1, the binding to isolated DR1 molecules is not even complete in 6 hours (T. Jardetsky, personal communication). It would therefore seem that the rapid binding to DR1 of influenza matrix peptide 17–29 seen with living cells is related to their biological activity and is not simply the consequence of the intrinsic high affinity of the system. Roosneck *et al.*[9] have seen that living B cells become fully competent to present a tetanus toxoid peptide to T cells after 40 minutes of exposure to it. Support for the concept that some activity of the living cells is necessary for the rapid loading of class II MHC with a peptide comes from experiments with glutaraldehyde-fixed EHM cells (Fig. 4), showing that these cells have lost the capacity for rapid binding of matrix peptide 17–29.

Fixation does not eliminate the class II MHC-dependent binding to the same cells when these are exposed to the peptide for 18 hours. This is in agreement with the previous report of Shimonkevitz *et al.*[7] showing that glutaraldehyde-fixed cells can stimulate T cells in a specific way when incubated in the presence of a peptide for 24 hours. It seems that the peptide-binding properties of class II MHC molecules in fixed cells are similar to those of the same molecules after purification. Figure 4 also shows that peptide binding by intact cells does not occur in the cold.

In conclusion, it seems that living B cells have a system for relatively rapid loading of class II MHC molecules with peptides. This system is probably not only operative at the level of the cell membrane, but also in intracellular vesicles where peptides derived from processing of internalized protein antigens are present. In this case, the MHC loading time must be shorter than the intracellular transit time of either the newly synthesized or the recycling class II MHC molecules. The molecular associations and structural events that make the rapid MHC/peptide associations in living B cells possible remain to be investigated.

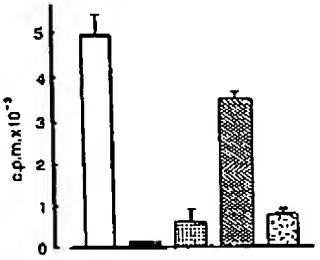

FIG. 4 Effect of glutaraldehyde fixation on binding of influenza matrix peptide 17-29-Tyr^{125}I. Cells were fixed with glutaraldehyde (Serva, Heidelberg) exactly as indicated by Shimonkevitz *et al.*[7]. They were then incubated for indicated periods of time at 37 °C with 10 ng peptide (in 0.1 ml medium with 10^6 cells). ▨, EHM cells, incubation 30 min; ▩, EHM cells incubation 18 h; flecked bar, MOLT 4 cells, incubation 18 h. In addition, the figure gives the data for unfixed EHM cells incubated for 30 min at 37 °C (open bar) or at 4 °C (filled bar). Every column gives the mean of triplicate cultures ± 1 s.d.

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A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin

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ANTIBODIES and growth factors have been chemically coupled to different toxins to produce cytotoxic molecules that selectively kill cells bearing appropriate antigens or receptors[1,2]. Antibody-toxin conjugates (immunotoxins) produced using conventional chemical coupling techniques have several undesirable characteristics. The smallest binding unit of an antibody is an Fv fragment which consists of a light and heavy chain variable domain. Recently, active single chain Fv fragments of antibodies have been produced in *Escherichia coli* by attaching the light and heavy chain variable domains together with a peptide linker[3,4]. Here we describe the construction and expression in *E. coli* of a single chain antibody toxin fusion protein, anti-Tac(Fv)–PE40, in which the varinble regions of anti-Tac, a monoclonal antibody to the p55 subunit of the human interleukin-2 receptor[5], are joined in peptide linkage to PE40, a modified form of *Pseudomonas* exotoxin lacking its binding domain. Anti-Tac(Fv)–PE40 was very cytotoxic to two interleukin-2 receptor-bearing human cell lines but was not cytotoxic to receptor-negative cells.

Immunotoxins made by chemically attaching a toxin to an intact antibody contain the constant region of the antibody, which is not necessary for immunotoxin action, but which reduces its access to target cells outside the circulation, and increases its immunogenicity. Furthermore, the product

heterogeneous and the yields are often poor. Recombinant DNA techniques have been used to produce chimaeric growth factor–toxin fusion proteins in *E. coli*[6–10]. These molecules can be readily purified in large amounts and contain only the sequences required for specific cell recognition and the cytotoxic activity of the toxin. Because the antigen-binding site of an antibody is composed of two separate polypeptide chains, it has been difficult to produce antibody-toxin chimaeric proteins in *E. coli*. To create a single-chain recombinant immunotoxin, we assembled a plasmid, pVC70108, which contains a 348-base pair (bp) DNA segment encoding an anti-Tac heavy chain variable domain (VH) joined to a 318-bp DNA segment encoding a light chain variable domain (VL) by a 45-bp linker; VL was in turn joined to a DNA segment encoding amino acids 253–613 of *Pseudomonas* exotoxin (PE) (Fig. 1). (The cloning and sequence of the anti-Tac variable regions will be described elsewhere.) The assembled gene is under the control of a T7 promoter[11]. The authenticity of the coding region of the plasmid was confirmed by DNA sequencing (data not shown). Upon induction with isopropyl-β-D-thiogalactoside (IPTG), BL21 (λDE3) carrying plasmid pVC70108 produced large amounts of a protein of relative molecular mass (M_r) ~65,000 (65 K), as shown by SDS-PAGE (lane 1, Fig. 2*b*). On immunoblots, the 65 K chimaeric protein reacted with an antibody to PE (data not shown). The fusion protein was mostly contained in the 100,000*g* pellet (lane 3, Fig. 2*b*) of the sonicated spheroplasts (lane 2, Fig. 2*b*). This pellet was used as the source to prepare anti-Tac(Fv)–PE40. Guanidine hydrochloride denaturation followed by rapid dilution was used to solubilize and renature the chimaeric protein[8]. The renatured protein was applied to a Mono Q column and the monomeric form of the fusion protein eluted at 0.2–0.22 M NaCl (Fig. 2*b*, lane 4 and Fig. 2*a*). High-molecular weight aggregates were eluted at higher ionic strength (fractions 42–50; Fig. 2*a*). Further purification of the chimaeric protein was carried out on a TSK-250 gel filtration column; the chimaeric protein eluted as a symmetrical peak at the location expected for a 65 K protein (data not shown). SDS-PAGE showed the protein to be >95% pure (lane 5, Fig. 2*b*) and N-terminal amino acid analysis showed the protein had the expected sequence, Met-Gln-Val-. Highly purified monomeric anti-Tac(Fv)-PE40 (~200 μg) was obtained from 1 litre of cells grown to an optical density at 650 nm of 0.6 before induction.

The anti-Tac antibody binds to the p55 subunit (Tac antigen, low affinity receptor) of the interleukin-2 (IL-2) receptor, which is present in large amounts on HUT-102 cells[5]. Therefore, the chimaeric protein was initially tested for cytotoxicity on HUT-102 cells. Anti-Tac(Fv)–PE40 inhibited protein synthesis in a dose-dependent manner with a 50% inhibitory dose (ID_{50}) of 0.15 ng ml^{-1} (2.3×10^{-12} M) in a 20 h assay (Fig. 3; Table 1). At concentrations >4 ng ml^{-1}, there was complete inhibition of protein synthesis. Several specificity controls were carried out. Addition of excess anti-Tac (10 μg ml^{-1}) prevented the cytotoxicity of anti-Tac(Fv)–PE40 on HUT-102 cells, whereas a control monoclonal antibody, OVB3, directed against an antigen found on ovarian cancer cells[12] did not (Fig. 3). The human T-cell leukaemia line Cr II.2 (ref. 13), which has a lower number

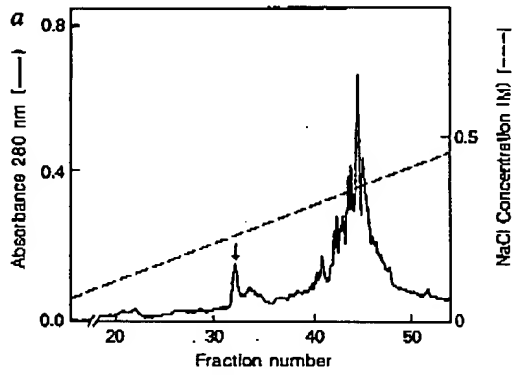

FIG. 2 Purification and characterization of anti-Tac(Fv)–PE40. *a*, Mono Q column chromatography of renatured soluble anti-Tac(Fv)–PE40. Renatured material was applied on a Mono Q column; proteins were eluted with a NaCl gradient (0–0.5M) and 4 ml fractions were collected. The position of active monomeric anti-Tac(Fv)–PE40 is shown by a vertical arrow. *b*, SDS-PAGE of samples at various stages of purification. The gel was stained with Coomassie blue. Lane 1, total cell pellet; lane 2, spheroplasts; lane 3, 100,00 *g* pellet of sonicated spheroplasts; lane 4, pool of fractions (32–33) from the mono Q column; lane 5, pool of peak fractions from the TSK-250 column; lane 6, native PE, M_r 66 K. Molecular weight markers (K) are indicated.
METHODS. *E. coli* strain BL21 (λDE3) carrying plasmid pVC70108 was grown, induced with IPTG, and the cell pellet was processed as described previously[8–9]. Dithiothreitol was omitted from denaturation buffer and renaturation was carried out for 16 h. After renaturation and dialysis, the sample was applied on a Mono Q column (HR 10/10) at 3 ml min^{-1}. The column was washed with 40 ml Buffer A (Tris-HCl 20 mM, pH 7.6) and developed with a 200 ml linear gradient (0–0.5 M NaCl). Eluted proteins were monitored at 280 nm. Fractions (4 ml) were collected and tested for cytotoxicity on HUT-102 cells. For SDS-PAGE, samples were boiled with Laemmli sample buffer[14] and electrophoresed on a 10% gel.

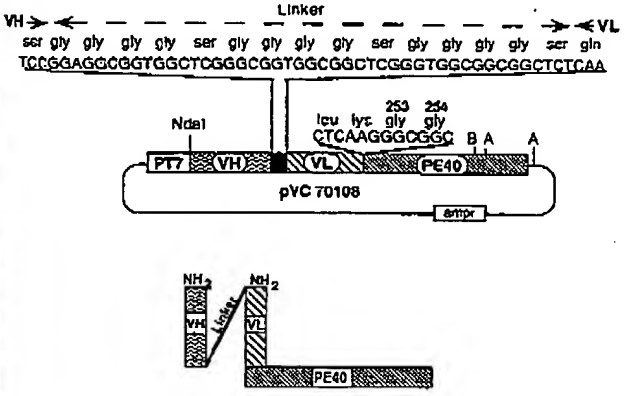

FIG. 1 a, Expression plasmid for anti-Tac(Fv)–PE40. Expression plasmid pVC70108 contains a fusion gene encoding various domains of anti-Tac (the variable domain of the heavy chain (VH, first 116 amino acids of mature heavy chain), a 15-amino-acid linker ((Gly-Gly-Gly-Gly-Ser)$_3$), and the variable domain of the light chain (VL, first 106 amino acids of mature light chain)) and amino acids 253–613 of PE (refs 6–9, 17) as a single polypeptide chain. The gene is under control of a T7 promoter linked to a Shine-Dalgarno sequence and initiation codon (PT7) as described previously[6–8,17]. *E. coli* strain BL21 (λDE3) carrying pVC70108 was used to express the chimaeric protein upon IPTG induction. Amp^r, β lactamase gene; B, *Bam*HI; A, *Ava*I. *b*, Schematic arrangement of various domains of anti-Tac(Fv)–PE40. The hybrid protein is shown as a single polypeptide chain. The C-terminus of VH is joined to the N-terminus of VL through a 15 amino-acid linker as shown in a. Details of the construction will be described elsewhere or can be obtained from the authors.

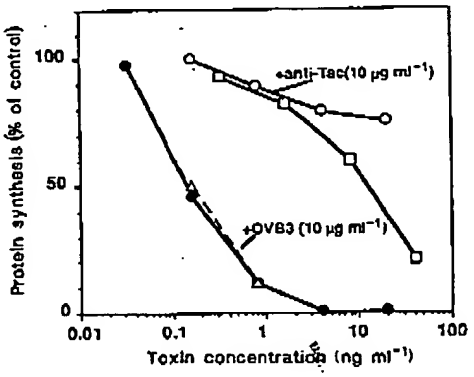

FIG. 3 Cytotoxicity of anti-Tac(Fv)–PE40 and anti-Tac-PE40 on HUT-102 cells expressing IL-2 receptors. Cytotoxicity was determined by measuring protein synthesis in HUT-102 cells after treatment with: (●) anti-Tac(Fv)–PE40; (○) anti-Tac(Fv)–PE40 +10 μg anti-Tac; (△) anti-Tac(Fv)–PE40 +10 μg OVB3; (□) anti-Tac–PE40.

METHODS. HUT-102 cells were washed twice with serum-free medium and plated in RPMI 1640 medium with 5% fetal bovine serum at 3×10^5 cells per well in 24-well plates. Various dilutions of recombinant anti-Tac(Fv)–PE40 and chemically conjugated anti-Tac-PE40 were prepared in PBS with 0.2% human serum albumin and added to appropriate wells. After 20 h the cells were labelled with [^{3}H]leucine for 90 min and the radioactivity in the TCA precipitate of the cell pellet determined. The results are expressed as % of control with no toxin added. For competition, 10 μg of anti-Tac or OVB3 were added to each well just before adding anti-Tac(Fv)–PE40.

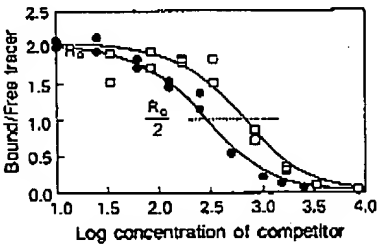

FIG. 4 Competition binding analysis of anti-Tac versus anti-Tac(Fv)–PE40. Competition of anti-Tac(Fv)–PE40 (open squares) and native mouse anti-Tac (solid circles) with [^{125}I] labelled tracer anti-Tac to bind to Tac antigen on HUT-102 cells is shown. Solid lines are computer generated idealized curves that model binding competition. Ro is the bound/free ratio for tracer in the absence of competitor, and Ro/2 is the 50% inhibition point for tracer binding, from which a binding affinity of 3.5×10^9 M^{-1} for anti-Tac(Fv)–PE40 is calculated, compared with 9.7×10^9 M^{-1} for native anti-Tac.

METHODS. [^{125}I]labelled anti-Tac (2 μCi μg^{-2}) as tracer was used at 1.5 ng per assay with varying concentrations of competitor and 4×10^5 HUT-102 cells as source of Tac antigen in 0.2 ml of binding buffer (RPMI 1640 with 10% fetal bovine serum, 100 μg ml^{-1} human IgG, 0.1% sodium azide), and incubated at room temperature with mixing for 2 h. Under these conditions, the concentration of tracer is 50 pM and Tac peptide 500 pM. Free tracer is 10 pM by calculation and satisfies the condition that free tracer be less than $1/K_a$=100 pM (using 10^{10} M^{-1} for anti-Tac K_a) for the assumptions of the competition analysis[19]. Assays were performed in parallel with a control cold anti-Tac antibody, and curve shifts at the 50% inhibition point of bound/free tracer binding (Ro/2) versus log competitor concentration were quantitated. The concentrations were obtained from the antilogs of the abscissa, and the affinity constant K_x for the construct, X, derived from the formula[19] $([X]_{1/2}-[\text{anti-Tac}]_{1/2})=1/K_x-1/K_a$ where $[\ ]_{1/2}$ indicates the concentration of competitor at which tracer binding is Ro/2. Standard Scatchard plotting of binding data with anti-Tac gave linear graphics and a K_a of 9.7×10^9 M^{-1}, comparable to that obtained by other investigators[16]. The K_x of 3.5×10^9 M^{-1} for anti-Tac(Fv)–PE40 was calculated from the above formula. All concentrations were measured by Bradford protein microassay against a standard curve with human IgG (ref. 20). For competition analysis, these concentrations were normalized on the basis of the bindable fraction obtained in separate tests with radiolabelled anti-Tac(Fv)–PE40 (0.44) and radiolabelled anti-Tac (0.8) with excess HUT-102 cells[16] to yield concentrations of bindable protein for the abscissa.

TABLE 1 Cytotoxicity of anti-Tac(Fv)–PE40 on various cell lines

Cell line	IL-2 receptors per cell: Low affinity	High affinity	ID_{50} ng ml^{-1}
HUT-102	94,000	3,800	0.15
Cr IL2	12,000	350	2.7
CEM	<20	<20	>1,000
OVCAR-3	—	—	>1,000
KB	—	—	>1,000
A431	—	—	>1,000

Cell lines OVCAR3, KB and A431 were seeded at 1×10^5 ml^{-1} in 24-well plates one day before the addition of toxin. HUT-102, Cr IL2 and CEM were washed twice and seeded at 3×10^5 ml^{-1} in 24-well plates (see also Fig. 3). RPMI 1640 with 10% fetal bovine serum was used for Cr IL2. Various dilutions of toxin preparations were added, and 20 h later the cells were labelled for 90 min with [^{3}H]leucine. The radioactivity in the trichloroacetic acid precipitate of the cells was determined. ID_{50} is the concentration of toxin that inhibits protein synthesis by 50% as compared with a control with no toxin added. All the assays were done in duplicate and repeated three times. (Data for the number of low and high-affinity IL-2 receptors on the various cell lines is from the unpublished data of T. Waldmann and ref. 16.)

of both low- and high-affinity IL-2 receptors than HUT 102, was also sensitive to anti-Tac(Fv)–PE40, with an ID_{50} of 2.7 ng ml^{-1} (Table 1). Furthermore, several human cell lines without IL-2 receptors, including the T-cell leukaemia line CEM, as well as carcinoma cell lines A431, KB and OVCAR-3 (ref. 14), were not affected by anti-Tac(Fv)–PE40, even at 1 μg ml^{-1} (Table 1).

Previously we reported that anti-Tac chemically conjugated to PE- or PE40-killed HUT-102 cells[14,15]. When thioether conjugates are made, anti-Tac-PE had an ID_{50} of 1.2 ng ml^{-1}, and anti-Tac–PE40 similarly prepared had an ID_{50} of 13 ng ml^{-1}. As anti-Tac(Fv)–PE40 (65 K) is about 30% smaller by weight than anti-Tac–PE (216 K), the chimaeric toxin is on a molar basis several times more active than anti-Tac–PE and considerably more active than anti-Tac–PE40. Anti-Tac–PE is a heterogeneous chemical conjugate in which the two molecules are connected by a thioether bond and different lysines in PE and anti-Tac are used in the conjugation reaction. In anti-Tac-PE40, the attachment appears to be mainly through the lysine residues in domain III of PE40 and this reduces the activity of the PE conjugates[14]. IL-2–PE40 is another chimaeric molecule, which was constructed by fusing a complementary DNA for human IL-2 to PE40 sequences[7]. IL-2–PE40 is slightly less cytotoxic to HUT-102 cells than anti-Tac(Fv)–PE40, with an ID_{50} of 1–5 ng ml^{-1}.

Competition binding studies showed an affinity of 3.5×10^9 M^{-1} for anti-Tac(Fv)–PE40, ~3-fold lower than that of anti-Tac, measured at 9.7×10^9 M^{-1} (Fig. 4). This can be compared with a fourfold loss of affinity of a Fv construct versus Fab fragment of anti-bovine growth hormone[3] and a sixfold loss for the Fv construct versus an Fab fragment of anti-digoxin[4]. The affinity of Fv binding for p55 seems to be preserved to a greater extent than similar preparations of anti-bovine growth hormone and anti-digoxin antibodies.

In summary, we have created an active immunotoxin in *E. coli* by the fusion of cDNAs encoding the anti-Tac variable regions with a fragment of DNA encoding a modified form of *Pseudomonas* exotoxin. Using this approach it should now be possible to create active recombinant immunotoxins with other antibodies. □

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Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein

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AN *N*-ethylmaleimide-sensitive fusion protein (NSF) has been purified on the basis of its ability to catalyse vesicular transport within the Golgi stack. We report here that this same protein is required for transport from the endoplasmic reticulum to the Golgi stack in semi-intact cells. This transport process is inhibited by a monoclonal antibody against NSF. Furthermore, pretreatment of semi-intact cells with *N*-ethylmaleimide, a sulphydryl alkylating reagent, inhibits transport. Addition of highly purified NSF largely restores transport from endoplasmic reticulum to Golgi. These results suggest that NSF is a general component of the transport machinery required for membrane fusion at multiple stages of the secretory pathway.

The *SEC18* gene of yeast encodes an NSF activity that will function in place of animal cell NSF with animal cell Golgi membranes[1]. As mutants in the *SEC18* gene are defective in transport from the endoplasmic reticulum (ER) to the Golgi in yeast[2] we wondered whether NSF is also required for this transport step in animal cells, in addition to its established role in promoting fusion within the confines of the Golgi stack. To address this issue we have examined whether NSF is needed for transport of the vesicular stomatitis virus (VSV)-encoded glycoprotein (G protein) between the ER and *cis* Golgi compartment in an *in vitro* system[3] in which semi-intact cells prepared from VSV-infected Chinese hamster ovary (CHO) cells are incubated in the presence of cytosol and ATP.

A complete incubation mix containing semi-intact cells, cytosol and ATP was treated with *N*-ethylmaleimide (NEM) on ice for 15 min (conditions which inactivate NSF[4]) before incubation. Subsequent transport (during an incubation at 30 °C for 90 min) was inhibited by more than 90 per cent (Fig. 1, lane iv). To determine whether the NEM-sensitive factor required for delivery of VSV-G protein from the ER to the *cis* Golgi compartment could be replaced by NSF, NEM-treated semi-intact cells and NEM-treated cytosol were incubated together at 30 °C in the presence or absence of NSF purified from CHO cells as described[5]. Addition of NSF stimulated transport about 10-fold over background (Fig. 1, compare lanes iv and v) restoring activity to a level which was nearly 50 per cent of that of an untreated incubation that had been supplemented with NSF (Fig. 1, lane iii). In this instance, NSF only marginally (10%) stimulated an incubation that had not been pretreated with NEM (Fig. 1, compare lanes ii and iii). But some preparations of semi-intact cells (apparently more deficient in NSF) are stimulated by up to 30 per cent (see Fig. 2). These results strongly

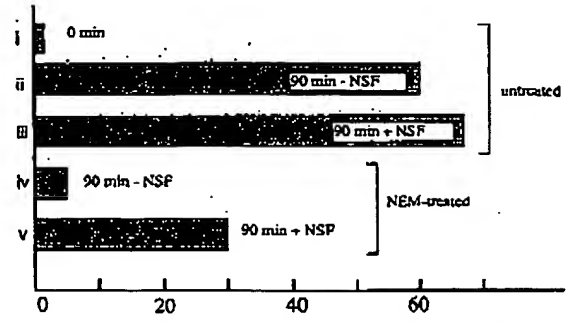

FIG. 1 NSF is required for transport from ER to Golgi. Assay conditions were as previously described[3,8]. Lanes i–iii, semi-intact cells, cytosol and ATP were incubated at 30 °C for the indicated time in the absence (lane ii) or presence (lane iii) of 0.16 μg of NSF (purified from CHO cells as described[5] in a final volume of 40 μl. Lanes iv and v, semi-intact cells and cytosol were pretreated with 1 mM NEM for 15 min at 0 °C. Subsequently, glutathione was added to a final concentration of 2 mM to quench unreacted NEM. NEM-treated membranes and cytosol were incubated in the presence of ATP at 30 °C for 90 min in the absence (lane iv) or presence (lane v) of 0.16 μg NSF. The fraction of total VSV-G transported was measured as the fraction of G protein in the Man_5 form as described previously[3,8].

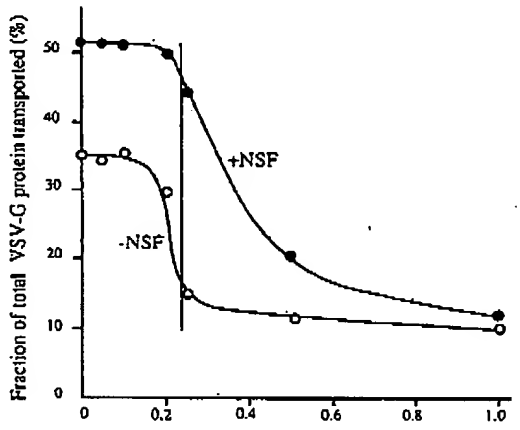

FIG. 2 Anti-NSF antibody inhibits the transport of VSV-G protein to the *cis* Golgi compartment. Assay conditions were as previously described[3,8]. The indicated amount of anti-NSF antibody was added to a 40 μl complete cocktail containing semi-intact cells, cytosol and ATP for 15 min on ice. Subsequently, the cocktail was transferred to 30 °C and incubated for 90 min in the absence (open circles) or presence of 0.16 μg purified NSF. The anti-NSF antibody (4A6) is an IgM whose properties have been described previously[5], and was purified from ascites fluid as described[5].

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